ACCESSORY GROWTH FACTORS FOR BACTERIA AND RELATED MICROORGANISMS

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A number of studies in bacterial nutrition have dealt with the so-called growth factors, accessory substances, vitamins, or growth activators,[1] substances which in small amount appear to play an important part in the development of certain bacteria

[1] The term hormone has also been used occasionally to designate these substances, but it does not seem to be valid on the basis of usage in mammalian and plant physiology.

and allied forms. The nature of these substances together with their possible function in the metabolic activity of various microorganisms has been a matter of conjecture for some time. Recently some interesting results have been secured which should lead to a much better understanding of the entire subject.

In the past some confusion has been caused by a rather indiscriminate application of similar terms to a variety of effects. In some instances the effect of an added substance has been merely that of stimulation, resulting in more abundant or more rapid development in an environment in which cell proliferation was already taking place. This stimulation has been due, at times, simply to adding more available food material to a "starvation" medium. In other cases, however, the added material has seemed to play a more important part in that its presence appeared to be necessary for development. At times very small amounts of added substance have permitted good growth in a medium in which the organism was not ordinarily able to multiply.

Substances which exert one or another of these effects have been found in a wide variety of extracts of plant and animal tissues. Through fractionation of these extracts attempts have been made to obtain the active components in pure form and to learn something of their chemical structure. The observations have been scattered over a rather wide field dealing with many miscellaneous sources of growth factors and with microorganisms of quite diverse groups. Before reviewing this material in more detail it seems best to consider briefly the basic ideas which have guided the investigational work.

It has usually been assumed that unknown chemical entities of organic nature were supplied by the added extract, and that these hypothetical substances produced the growth-promoting effect. Other interpretations have been advanced from time to time. These can be summarized as follows: (1) The added material may have supplied certain needed inorganic salts, particularly those of metals which act as catalysts; (2) the added material may have combined with and removed from activity

an excess of some constituent which was present originally in sufficient concentration to be toxic; or (3) it may have altered the physical characteristics of the medium so that cell proliferation became possible. Sole reliance on any of these explanations makes unnecessary the assumption of the existence of essential accessory substances of organic nature.

At the present time we appear to have reached a point from which a clearer idea of these effects may be gained. In a few instances it has been found possible to substitute, either fully or in part, known chemical compounds for the indefinite growth-promoting entities of tissue extracts. Eventually we should be able to abandon the use of such terms as V and X factors, L fractions, and others of like nature as their specific identities become established.

It seems best to review first the fractionation of tissue extracts and other sources, pointing out those instances where the active substance has been identified or where a knowledge of its properties has progressed to such a point that its nature could be surmised and a known compound substituted successfully for it. A consideration of the other explanations of growth-factor activity will follow. No attempt will be made to review the earlier work, other than that needed as a background for the present discussion, since former studies have been summarized by Knorr (56), Sergeant (142), Peskett (111) and Knight (52). Also, no attempt will be made to treat the subject of plant auxins. Various bacteria and other fungi are important in the production of substances, such as 3-indoleacetic acid, which exert a marked influence on plant cell elongation and multiplication. So far as the writers are aware, however, there is no instance on record as yet in which auxin-*a*, auxin-*b*, 3-indoleacetic acid or related compounds have proved to be essential for cell proliferation of bacteria. The production of these substances by bacteria and their effects on plants is outside the scope of this article and readers are referred to the recent publications of Boysen-Jensen (6), Schlenker and Rosenthal (128), Went and Thimann (164), Nicol (101) and others.

EXPERIMENTAL PROCEDURES

In experimental work dealing with the growth accessory factors for microorganisms details of technique are quite important. Although these items have been mentioned from time to time in studies on bacterial metabolism, often they have been neglected, and so it seems desirable to emphasize again certain points.

Sterilization of the fractions to be tested for growth-factor activity presents a problem at the outset. The unknown substances in tissue extracts may be destroyed by heat, while resort to filtration may cause serious loss due to inactivation or adsorption. The checking of one method against the other is useful, though there is the possibility that some of the factors may be inactivated by both procedures.

Basic medium for the tests. In tests for growth accessory factors, the basic medium should be adequate in all other respects and the conditions of cultivation should approach as closely as possible those known to be most suitable for the organism in question. Unfortunately our knowledge of these requirements is notoriously inadequate. Concerning many microorganisms little is known of their actual needs with respect to various amino acids and other nitrogenous ingredients, the inorganic salts and their proper physiological balance, and other factors such as osmotic pressure, surface and interfacial tension, gaseous environment, redox potential and kindred conditions. Consequently our efforts are often seriously hampered at the start, and it is uncertain whether these important conditions are being satisfied. To avoid the uncertainties regarding amino acid requirements, some investigators have used hydrolyzed casein or gelatin as a basic medium. This procedure, however, is subject to the disadvantage of introducing impurities with the casein and gelatin, and also in that the medium is no longer of known composition.

Size of inoculum. Inoculation of the test medium with large numbers of cells may introduce appreciable amounts of growth factors, either from the cells themselves or from the previous culture medium. While this added material may be eliminated by several successive transplants in the new environment, in

general it would appear to be more desirable to start with smaller numbers of cells, thus affording at the outset a stricter test of the ability of the cells to multiply in the new medium.

Storage of growth factors in cells. If cultures are grown in a medium containing an excess of some required factor, they may possibly store sufficient quantities of the material to permit of further limited proliferation when transferred to a deficient medium. This could well lead to erroneous conclusions or at least make difficult the interpretation of results.

Multiple growth requirements. Tissue extracts contain many biologically active substances. Liver, for example, has been shown to contain a number of the accessory substances as well as amino acids. Therefore in attempting to isolate growth substances for microorganisms due consideration must be given the multiplicity of possible factors. This is well exemplified in the work on bios. It must be borne in mind too that amino acids frequently accompany accessory factors through the earlier stages of attempted separation. If one or more of these amino acids is essential for growth and if it is not supplied in the basic medium, its subsequent removal during chemical manipulation will give rise to a deficient environment and the organism under investigation will be unable to multiply for this reason rather than because of a lack of other needed substances.

Methods of assay. Of the several methods which have been proposed for the quantitative determination of the potency of growth-factor preparations, by far the most widely used is that of visual inspection of the culture tubes for turbidity. This method has a large error but is simple and rapid. The use of a cell and thermocouple for the determination of the density of the suspension probably increases the accuracy of this method (169). More precise results can be obtained by direct count but where a large number of determinations is to be made, the time required would be a serious disadvantage. Direct weighing of the mass of organisms formed has been employed. In the case of the mycelium of molds (16) weighing probably gives accurate results, but with bacteria and yeasts it is doubtful whether the method is much more accurate than visual inspection. Indirect

determination (92) of the mass of bacterial growth by means of its nitrogen content is probably more accurate than inspection, although subject to some rather serious errors due to the possibility that nitrogenous material from the medium might be included with the organisms, or if the organisms are washed too thoroughly nitrogen may be lost. Sternfeld, Wermuth and Saunders (148) attempted to follow growth by means of changes in conductivity, refractive index and other physical properties of the cultures, but the differences were too slight to be useful. Some workers (50, 144) have used the titer of acid formed by bacteria as an index of growth.

In the following sections discussion of the growth-promoting materials for bacteria and the related non-chlorophyll bearing fungi has been arranged under the different groups of microorganisms.

VARIOUS GROUPS OF THE BACTERIA

The streptococci and allied coccus types

The nutritive requirements of the pathogenic streptococci have always been quite obscure. These organisms practically without exception fail to develop in various amino acid synthetic media (40, 64, 67, 29) and attempts at separation of essential growth substances from meat infusions or other similar sources seem to have been attended by unusual difficulties. In a few instances the active material has been carried through several preliminary stages in the process of purification but little success has been attained beyond this point.

By adsorption with fuller's earth and charcoal, Freedman and Funk (37) obtained from beef infusion, autolyzed brewer's yeast and peptone, substances which showed growth-stimulating activity for hemolytic streptococci. Substances with a similar growth-stimulating effect were also found on hydrolysis of certain proteins, particularly casein, commercial gelatin, yeast protein and edestin (37). The evidence indicated that the active substances were not constituents of the protein molecule itself. Mueller (91) showed that wood charcoal removed from beef heart infusion some component needed for development of the

streptococcus. The infusion could be reactivated by addition of small quantities of peptone or acid hydrolysates of casein and edestin. The activating material was separated by precipitation with heavy metals into two fractions which exhibited activity only after mixing.

Whitehead (165) applied precipitation with phosphotungstic acid to a tryptic digest of casein. Substances necessary for growth of a hemolytic streptococcus were removed with the precipitate but were not effective in supporting growth unless small quantities of the filtrate were also added. A further separation was accomplished by extraction with butyl alcohol. Hosoya and Kuroya (47) reported that an alcoholic extract of rice bran supplied something needed by hemolytic streptococci and that this material accompanied vitamin B. McLeod and Wyon (85) attempted to determine the property of fresh blood and serum which promoted growth of pneumococci and meningococci. This property of serum could not be extracted by butanol, and digestion of serum with trypsin destroyed it. They believed the effect of serum was a phenomenon of the colloidal state. Recently Rane and Subbarow (114a) reported that a mixture of glutathione, thiochrome, flavin, nicotinic acid, betaine, glucosamine and a calcium-alcoholic precipitate of highly purified liver extract, in a deficient basal medium, provided almost optimum conditions for growth of the Dochez NY5 strain of hemolytic streptococcus. Omission of one or more of these factors decreased the amount of growth.

The saprophytic streptococci, particularly those of importance to the dairy industry, have also received some attention. Orla-Jensen, Otte, and Snog-Kjaer (108) found that the active material in skim milk could be removed by adsorption on charcoal or fuller's earth and elution with a methanol-pyridine solution. This growth-promoting activity appeared to be due to several factors, one of which could be replaced by riboflavin. Wood, Andersen and Werkman (175) reported that growth of *Streptococcus paracitrovorous* was improved by the addition of riboflavin.

Working with several representative streptococci, Hutner (50) found that at least one factor could be removed from depro-

teinized milk by adsorption with certain brands of fuller's earth. The growth-promoting activity thus removed could not be replaced by the addition of pure compounds such as thiamin, riboflavin, uracil or guanine. The finding with respect to riboflavin is contrary to that of Orla-Jensen. Rahn and Hegarty (114) noted that lactic acid production by centrifuged and washed cells of *Streptococcus lactis* was increased regularly by the addition of 0.002 per cent nicotinic acid. Small amounts of ascorbic acid stimulated injured or exhausted cells. Adenine, inositol and riboflavin produced no effect. In several of these studies lactic acid-producing streptococci have been used along with the lactobacilli. A further consideration of this work is presented in a later section dealing with the *Lactobacillus* group.

Knowledge of the growth-accessory factors for the streptococci has in general not progressed much beyond the stage of impure tissue extracts. Although there have been isolated reports of the effect of chemically pure compounds, their efficacy in promoting growth of the various types of streptococci is not established at present.

Staphylococcus

On fractionation of meat extract, Hughes (49) obtained an "activator" for staphylococci. This was effective in promoting growth in Uschinsky's medium or in a casein digest medium, in which freshly isolated strains were incapable of multiplication. The active material was concentrated to a point where the addition of .0001 milligram to 5 cc. of casein digest supported ready development. It was heat stable at pH 7.0, soluble in water, alcohol and acetone, but insoluble in ether and benzene. It dialyzed through collodion membranes and disappeared on acid hydrolysis of the meat extract. An apparently similar material was obtained from yeast extract ("marmite") by Knight (51). Typical strains of *Staphylococcus aureus* grew readily upon the addition of small amounts of this material to a basal medium of hydrolyzed gelatin, amino acids and glucose.

Further studies of this fraction by Knight, Fildes and associates have been instrumental in throwing light on the real nature of the active materials and their work constitutes an important

contribution to our knowledge of essential nutritive substances for bacteria. A high-vacuum distillate containing the active growth factor was used for further analysis. Biological indications from other sources, together with chemical and spectrographic evidence of their own, suggested the testing of cozymase, nicotinic acid, nicotinamide, and thiamin as definite compounds to replace the unknown yeast factor. Later, in a study of the absorption spectrum of the high-vacuum distillate secured from yeast, Holiday (45) concluded that nicotinic acid was present in the free state in the yeast concentrate.

It was found that the factor was a complex and that one component of it could be replaced by nicotinic acid (or nicotinamide) and the other by thiamin (Knight, 53). These two substances were not effective when added singly, but when supplied together a ready development of *Staphylococcus aureus* was secured. On substituting a collection of amino acids for the gelatin hydrolysate previously used, the organism was then grown in a medium the constituents of which were chemical entities of known structure (Fildes et al., 36).

The small amounts of nicotinic acid or its amide and of thiamin which sufficed for development were quite striking (Knight, 53, 54). A concentration of the amide of 6.6×10^{-7} M (0.08 microgram per cubic centimeter of medium) supported maximum growth in 27 hours in the synthetic medium, while light but still detectable growth was secured in the presence of 2.6×10^{-8} M amide. The smallest amount of thiamin which supported maximum development was about 1.0×10^{-8} M while 5.0×10^{-10} M produced a detectable effect. These amounts are equivalent to 0.003 and 0.00015 microgram per cubic centimeter of medium, respectively.

The activity of compounds related to thiamin was also studied by Knight (54, 55a). The components of the thiamin molecule, namely the pyrimidine plus the thiazole, were effective in place of the complete molecule (in the presence of appropriate amounts of nicotinamide). However, a number of other closely related compounds could not be substituted, indicating a high degree of specificity in the requirement of this organism.

For anaerobic growth of the staphylococcus, Richardson (121)

reported that uracil was required, a concentration of M/20,000 being most effective. Twenty-one other related compounds were studied but showed no comparable effect. This striking specificity of uracil, it was suggested, indicates that the compound must be widely distributed in nature and that it exerts an effect which cannot be reproduced by adenine and its derivatives.

Another item concerning growth of the staphylococcus has been added by van Wagtendonk (cited by Kögl, 57). The addition of Kögl's "biotin" in the form of its methyl ester resulted in more luxuriant growth. Amounts of 0.005 and 0.05 microgram of the biotin ester produced a three- to four-fold stimulation of growth when added along with small amounts of thiamin and nicotinic acid. Biotin ester alone gave a slight increase in growth. In this instance a compound has been added which has been obtained in crystalline form although the chemical structure is unknown.

These results indicate that for best growth of at least some strains of staphylococci, something more than thiamin and nicotinic acid is needed. Since Knight reported very good growth of his *Staphylococcus aureus* in approximately 24 hours, it is possible that some strains may not need biotin or are able to synthesize it themselves. In a recent confirmation of Knight's work, the writers and associates (63) found that a strain of *Staphylococcus albus* developed in a synthetic medium containing thiamin and nicotinic acid, but growth was considerably slower than that secured in broth or after the addition of fractions of a spleen preparation to the synthetic medium. Evidently something else was needed by this strain for optimum growth, though whether this need could be filled by biotin is not known.

Whether or not another substance is needed for best growth of the staphylococcus, it has been demonstrated by Knight that this organism, which formerly failed to grow in synthetic media, can now be grown successfully by the use of chemically definite compounds. It is of interest to note that of the growth factors required by the staphylococcus, the two for which the chemical structure is definitely known (thiamin and nicotinic acid) are also needed for the normal functioning of the mammalian organism.

The diphtheria bacillus

The nutritive requirements of this organism have been the subject of many studies. Most strains refuse to grow in synthetic media composed of the usual amino acids, salts and sugar. Evidently something else is needed for development. Separation and identification of these additional growth factors have been the objectives of an interesting series of reports by Mueller and associates. Starting with a suitable medium containing either meat extract or other extracts of fresh tissues, they attempted to separate the components needed for cell multiplication (98, 93). Most of the growth-promoting activity of liver preparations was found in an alcohol filtrate of an aqueous extract, and substances essential for growth could be removed from such a solution by adsorption with wood charcoal and later recovered from the charcoal by elution with acid alcohol (93).

Further studies of the liver eluate by Mueller showed that the active materials could be separated into two fractions by repeated extraction of acid solutions with ether. Both fractions were required for the full growth-stimulating effect (99). The ether-extractable substance from liver could be replaced by concentrates of urine (cow and horse). On further investigation of this source and the use of fractional distillation with the Rittenberg apparatus an active substance was obtained and identified as pimelic acid, $C_5H_{10}(COOH)_2$ (94).

When added to a basic medium consisting of casein hydrolysate, cystine, glutamic acid, sodium lactate and inorganic salts together with the ether-insoluble liver fraction, either the isolated product or synthetic pimelic acid produced a two- to three-fold stimulation of growth. Quantitative determinations of bacterial nitrogen (92) showed that the stimulating effect of pimelic acid became evident at a concentration of about 0.005 microgram per cubic centimeter of medium and reached a maximum in the presence of five to ten times this amount (94). Other dibasic acids of the same series, from oxalic up to azelaic, exerted no growth-stimulating effect.

Following the identification of pimelic acid, attention was next turned to the ether-insoluble fraction of liver extract. This

material, after combined esterification and acetylation, was subjected to fractional distillation; and growth-promoting activity appeared in both the lowest-boiling and the highest-boiling fractions. Nicotinic acid was substituted for the low-boiling fraction and showed the same growth-promoting activity. The most striking effect of nicotinic acid was exerted in a concentration of about 1.0 microgram per cubic centimeter of medium while approximately ten times as much nicotinamide was required to produce a comparable effect (95).

The high-boiling fraction of the vacuum distillate remained as the only source of unidentified material and this was next subjected to examination by Mueller and Cohen (97). Chemical evidence indicated the presence of amino acids and it was found that β-alanine, which had been shown by Williams and Rohrman (171) to exert a growth-promoting effect on yeast, could be substituted for the high-boiling material. β-Alanine produced its maximal effect in a concentration of about 1 microgram per cubic centimeter of medium. *l*-Carnosine (β-alanyl histidine) was also effective but a greater concentration was required (96).

As a result of these studies it was shown that three substances of known chemical structure could be substituted for the hitherto unknown materials in extracts of liver and other tissues. In comparing the effect of these three compounds on the growth of four strains of diphtheria bacilli it was evident that each of them, when supplied alone in the basal casein-hydrolysate medium, exerted no appreciable growth-promoting effect. β-Alanine and nicotinic acid together were quite effective, and for some strains pimelic acid exerted an added stimulative effect (97). On substituting amino acid mixtures for the basal medium of hydrolyzed casein, several cultures of the Park 8 strain developed readily and produced a potent toxin in a medium of definite chemical composition (110). These reports by Mueller and associates provide an excellent example of the value of intensive and thorough search for the substances in tissue infusions which are required by some bacteria; and they constitute an important contribution to our knowledge of the nutritive requirements of bacteria.

The rôle of β-alanine and nicotinic acid was recently confirmed by other workers (63) though β-alanine appeared to be the more important of the two substances insofar as one Park 8 culture was concerned. Addition of β-alanine alone to a basal medium of amino acids, dextrose and mineral salts resulted in growth of a strain of the organism which failed to develop without the β-alanine. In contrast, nicotinic acid or pimelic acid alone did not support growth but the former stimulated development when added with β-alanine.

While Mueller's work has done much to clarify our knowledge of the usual requirements of this organism, it should be added that apparently some strains of diphtheria bacilli either do not require the foregoing compounds or else are able to synthesize them, for evidence has appeared from time to time that occasional strains of the organism may be cultivated in ordinary amino acid synthetic media and in this earlier work β-alanine and nicotinic acid were of course not used. Typical of such reports are those of Braun, Hofmeier and Mundel (7), of Maver (84) and of Wadsworth and Wheeler (162). The last mentioned investigators obtained growth of thirteen out of twenty recently isolated virulent strains in a synthetic medium which did not contain β-alanine and nicotinic acid. Development of the cultures was slow, but could be carried through successive transplants and weak toxin was produced.

Dysentery bacilli

Many strains of dysentery bacilli fail to develop in the usual synthetic media composed of amino acids, glucose and inorganic salts; evidently other substances or conditions are required. Upon addition of small amounts of tissue extracts to such a medium, the cultures usually develop readily. The growth-promoting substances in veal infusion, yeast, and other animal and plant tissues can be obtained in impure form by charcoal adsorption (65). They can also be partially purified by treatment of tissue infusions with solutions of heavy metals which precipitate inert material, but do not precipitate the growth factors (127).

Recently it has been shown by Koser, Dorfman and Saunders

(61) that nicotinic acid or nicotinamide can be substituted for the fractions from tissue extracts and thus it is now possible to secure growth in a medium of definite chemical composition. Amounts of 0.1 microgram of nicotinic acid per cubic centimeter of synthetic medium caused prompt growth with pronounced turbidity of a number of Flexner and Sonne strains, while 0.01, 0.004 or at times even 0.002 microgram per cubic centimeter sufficed for slower and scantier development. Whether the growth-promoting property of the tissue extracts is due to nicotinic acid or to the amide is not known, since the presence of these compounds has not yet been definitely established in these preparations.

It is of interest that for the dysentery bacilli nicotinic acid, or its amide, seems to be the only substance needed in addition to amino acids, glucose and salts. In the case of the staphylococci, it will be recalled, both nicotinic acid (or the amide) and thiamin were needed, and for the diphtheria bacillus a combination of nicotinic acid and β-alanine gave the best results.

Brucella

Growth-promoting substances in extracts of yeast and beef liver were precipitated with phosphotungstic acid and found to be remarkably stable on heating in the presence of acid or alkali (48). Koser and Saunders (65) found that growth-promoting activity for *Brucella* could be removed from various plant and animal tissue extracts by charcoal adsorption and recovered by subsequent elution with alcohol or acetone. The active substances could also be concentrated by precipitation of inert material with heavy metals. Substitution of various definite compounds for the active fractions of tissue extracts has not been successful. Thus, the addition of nicotinic acid, thiamin, riboflavin, β-alanine and other compounds to a synthetic medium was not followed by growth of a *Brucella abortus* culture (63).

Development of some *Brucella* cultures in synthetic media without the addition of added growth factors has been reported by ZoBell and Meyer (179), though even the most promising of their synthetic media were far from satisfactory. Growth was slow and some cultures refused to multiply in the second trans-

fer. Furthermore, from one hundred thousand to a million cells per cubic centimeter of synthetic medium were needed to insure positive results. The inability of small numbers of cells to initiate growth suggests that other factors or conditions were needed.

The hemophilic bacteria

Studies of the accessory requirements of *Hemophilus influenzae* and allied hemophilic types have received more attention and are better known to most bacteriologists than those dealing with other groups of microorganisms. The reports of Davis (20, 21), Thjötta and Avery (153, 154, 155), Fildes (32) and others demonstrated that two substances were necessary for development of Pfeiffer's bacillus. One of these was associated with the hemoglobin of blood and the other occurred in a variety of plant and animal tissues or in the extracts of microorganisms. Extracts of potato apparently contained both substances. From these studies there emerged the now-familiar V and X factors. Separately these factors are not sufficient for cell proliferation of *H. influenzae* but when supplied together in ordinary culture media prompt development occurs.

The V factor is thermo-labile, diffuses through parchment membranes and is easily destroyed in alkaline solution. Its potency is lowered or it may be completely inactivated on contact with fresh serum. It is produced by a number of bacteria as well as by yeasts and molds and is found in many plant and animal tissues. The X factor is relatively thermo-stable and is associated with the iron-containing fraction of hemoglobin. It may be replaced by hematin. It occurs in plant tissue, especially potato, and is probably elaborated by some bacteria. It is often, though not always, associated with peroxidase activity and it has been suggested by several workers that its action is of a catalytic nature, accelerating the transfer of oxygen from peroxides in the medium or from the atmosphere to the bacillus. The essential points with respect to the V and X factors were confirmed and extended by a number of workers during the several years following 1921. No detailed account of these

results need be given here since this material has been covered in previous reviews (141, 111, 52).

The requirements of other organisms of the influenza group have been studied to some extent. Certain representatives of this group required only the V factor and were termed *H. parainfluenzae* by Rivers (123). Among these influenza-like bacilli there were encountered both hemolytic and non-hemolytic strains which possessed the common characteristic of being able to develop in the presence of the V but without the X factor (33, 160). In contrast to these organisms are the so-called *B. hemoglobinophilus canis* of Friedberger which requires only the X factor (Rivers, 122) and Ducrey's bacillus of soft chancre (*Hemophilus ducreyi*), which according to Lwoff and Pirosky (79) needs for its growth the X factor (hemin) but not the V factor.

More recently some additional information on the nature of the V factor has appeared. The earlier suggestions that the V factor might be vitamin C (ascorbic acid) appear to have been definitely ruled out by Meyer (86). Studies on the coenzyme of Warburg and the cozymase of Harden and Young have afforded the basis for an important step in our understanding of the V factor. Lwoff and Lwoff (76) found that either the coenzyme or the cozymase could be substituted for the unknown V factor and *H. parainfluenzae* then developed readily in peptone solution. It is interesting that nicotinic acid, its amide, diethylamide and adenylic acid could not be substituted for the coenzyme or V factor. This represents a contrast to the staphylococci and the dysentery bacilli which either are able to synthesize the codehydrogenase or can utilize the constituent parts as such.

It was found also (77) that the codehydrogenases had no influence upon the speed of reduction of methylene blue or of oxygen uptake by cells of *H. parainfluenzae* grown in the presence of an excess of V factor, but did increase these processes by cells grown in the presence of small amounts of V factor. When approaching the limit of active dilutions, the action of the codehydrogenases was quantitative. Evidently the physiological function of V factor is that of a catalyst in cell oxidations. Similar evidence was submitted by Lwoff and Lwoff (78) with respect to

the rôle of hemin (X factor). They believe that the function of hemin as a growth factor is in the formation of respiratory enzyme systems such as cytochrome, cytochromoxidase, catalase and peroxidase. These interesting contributions supply evidence concerning not only the chemical nature of the hitherto mysterious V factor, but also the rôle of both the V and X factors.

Acid-fast bacteria

Johne's bacillus usually fails to develop even in the more complex media, unless killed cells or extracts of other acid-fast bacteria are added. Twort and Ingram (157) attempted to isolate the substance essential for Johne's bacillus from cells of other acid-fast types, such as *Mycobacterium phlei.* Several extracts were prepared and from one of them a small amount of the active substance was precipitated with barium salts. Further purification was not attained. The growth-promoting activity was not destroyed by autoclaving. While it is uncertain whether the so-called "essential substance" studied by Twort and Ingram over twenty-five years ago can be classed with the accessory factors, the work nevertheless possesses considerable interest as being one of the earliest attempts to isolate growth substances from complex mixtures.

Tubercle bacillus. Of the various pathogenic bacteria the tubercle bacillus is often regarded as one of the less exacting in its nutritive requirements, since many strains may be grown in the simpler synthetic media, of which Long's is probably the best known. There is evidence, however, that additional substances are required for maximum development and that the tubercle bacillus is unable to initiate growth in Long's medium unless the solution is seeded with large numbers of cells. Uyei (159) states that growth in Long's medium occurred only when the inoculum contains 1 milligram of cells (about five billion), whereas in Petroff's glycerol-egg medium and in a potato-glycerol medium development of cultures could be secured with inocula of 0.001 milligram and 0.000,000,001 milligram, respectively.

Addition of a yeast preparation or of orange, tomato or cabbage juice to Long's medium increased markedly the amount of growth

of both human and bovine types of the bacilli after 2 and 3 weeks of incubation (Uyei, 158). Of the several interpretations which may be made from such an observation, Uyei emphasized the supposed vitamin-like nature of the accelerating substances and suggested a relationship to vitamin B (complex). Uyei (159) also studied the nature of the growth-promoting principles of the potato and reported that the active substances could not be extracted with acetone, alcohol, or ether.

Not only is the nature of growth accessories for the acid-fast organisms unknown, but there is doubt as to whether such substances are required for the better-known representatives of the group.

The anaerobic spore-formers: genus Clostridium

A "vitamin" necessary for cell proliferation of *Clostridium sporogenes* was described by Knight and Fildes (55). Cultures of the anaerobe failed to develop in an acid hydrolysate of photographic gelatin supplemented by tryptophane, sodium citrate, thioglycollic acid and inorganic salts unless small amounts of the "sporogenes vitamin" were also added. Two-tenths of a microgram in 10 cc. of basal medium was sufficient for growth just visible to the eye. The active material was obtained in the form of a yellow gum from yeast and could also be obtained from human urine. The same substance, or something capable of replacing it, was also synthesized by certain microorganisms, notably *Salmonella aertrycke*, the tubercle bacillus, and *Aspergillus versicolor*.

Substitution of an amino acid mixture for the gelatin hydrolysate was made possible through the study of Fildes and Richardson (35) and development of *Cl. sporogenes* was then secured in a medium the only unknown component of which was the "vitamin." Fildes (34) has presented evidence to show that the "sporogenes vitamin" is also needed by many strains of *Cl. botulinum*. Pappenheimer (109) made a further study of the chemical properties of this growth factor. Highly active preparations were secured but the substance could not be obtained in crystalline form. The material had the properties of an unsaturated hy-

droxy-acid of molecular weight about 200, and the formula $C_{11}H_{14}O_4$ or $C_{11}H_{11}O_4$ was suggested. The factor was considered to be distinct from the plant auxins, Williams' pantothenic acid, the staphylococcus factor of Hughes, and the bios of Kögl.

Propionic acid bacteria; lactobacilli; butyl alcohol bacteria

Propionic acid bacteria. These microorganisms are usually considered to be fastidious in their growth requirements since they do not multiply readily in the ordinary peptone medium, but develop much more rapidly upon the addition of milk whey, yeast extracts or tissue extracts. Van Niel (161) believed the superior fermentation obtained in the presence of yeast extracts and yeast autolysates could not be ascribed to differences in nitrogen content or buffer capacity and suggested that accessory substances in yeast might play an important part.

The stimulative effect of potato extract, orange juice and yeast-water on glucose fermentation and acid production by these organisms was studied especially by Fromageot and Tatum (38) and by Tatum, Peterson and Fred (150). Evidence indicated that the stimulative activity of potato extract was not due primarily to available nitrogen content or buffering capacity. By use of the Neuberg reagent (mercuric acetate and sodium carbonate) the potato extract was separated into two fractions both of which were needed for maximum stimulation. The Neuberg filtrate fraction was believed to contain some accessory substance other than mineral salts, since after ignition the ash did not produce the complete stimulative effect (150). The effect of the Neuberg precipitate was due primarily to ammonium nitrogen and asparagine. Ammonium nitrogen was utilized in the presence of the proper growth factors (151).

In a continuation of this work, yeast extract was used as the source of growth stimulant and from it Wood, Tatum and Peterson (176) obtained a fraction apparently essential for growth of various strains of propionic acid bacteria in a glucose-ammonium sulphate medium. This factor was acidic in nature, non-volatile and could be extracted with ether. It could not be replaced by other biologically active substances, namely thiamin,

the flavin fraction from liver, the sporogenes vitamin of Knight, Williams' pantothenic acid, indoleacetic acid, inositol, or nicotinamide. It should be emphasized, however, that the propionic cultures did not grow indefinitely in the glucose-ammonium sulphate-yeast factor medium, indicating the need for some additional material. This was supplied by hydrolyzed casein or by unhydrolyzed casein, egg albumin or milk powder. From these sources, as well as from yeast extract, the active material could be extracted with alcohol and acetone. It was neither an amino acid nor a part of a protein molecule (Tatum, Wood and Peterson, 152). The solubilities and stability of the active fraction resembled those of thiamin and this similarity suggested substitution of the pure vitamin. Two different samples of thiamin were found to be capable of completely replacing the extract. One sample was effective in amounts of 0.005 microgram per cubic centimeter of medium while 0.05 microgram of the other lot was required. Inositol, pantothenic acid, ascorbic acid, hepatoflavin, nicotinamide and indoleacetic acid were not effective in replacing the extracted material. Here we have an interesting instance of the replacement of an unknown growth-stimulating material by a known substance of definite chemical composition, thus advancing materially our knowledge of the physiological requirements of the propionic acid bacteria. Evidently, at least one other substance is needed and it is contained in the acid-ether extract of yeast or potato.

There is also evidence that riboflavin is a stimulant for propionic acid bacteria. On fractionating yeast extract, Lava, Ross and Blanchard (69) found the B_2-containing portion to be the most active in stimulating acid production. This was confirmed with pure riboflavin by Wood, Andersen and Werkman (175). They also found (175a) that the factor in the ether extract of yeast extract was essential for all cultures of propionic acid bacteria. This factor could not be replaced by a mixture of nicotinic acid, thiamin, pimelic acid, uracil, β-alanine and "pantothenic acid." Riboflavin and thiamin stimulated growth but were not essential.

Lactobacilli. Orla-Jensen, Otte, and Snog-Kjaer (108) stated

that riboflavin and one or more other "activators" are necessary for normal development of certain lactic acid bacteria. Their conclusion that one of these substances is pantothenic acid seems questionable, however. Their finding concerning riboflavin was confirmed (175). Other unknown substances were also needed and the requirements varied somewhat with different lactic acid types. Unknown factors in the basal medium (175) were the ether-soluble component from yeast and hydrolyzed casein. Seventeen purified amino acids did not satisfactorily replace the hydrolyzed casein.

Snell, Tatum and Peterson (145) reported that two unknown factors appeared to be necessary for attainment of luxuriant growth by *Lactobacillus delbrückii* in a hydrolyzed casein medium containing added tryptophane and a fermentable carbohydrate. One of these factors occurred in the Neuberg filtrate fraction or in an acid-ether extract of crude potato extract. The evidence suggested an acid of fairly low molecular weight. The second factor occurred in peptone, was basic and could be precipitated with Neuberg's reagent and with lead acetate and ammonia. Liver extract contained both of the growth stimulants, or other substances capable of replacing them.

In a later report Snell, Strong and Peterson (144) found one of the factors in liver to be an acidic, ether-extractable organic substance. The maximum effect of this fraction was attained in the presence of 0.1 to 0.3 microgram per cubic centimeter of basal medium, though its effect was detectable with amounts as small as 0.003 microgram. The basal medium contained riboflavin, which also exerted a stimulating effect in small amounts, and sodium acetate in addition to other more commonly used substances. A number of known compounds were tested but failed to replace the fraction from liver. These were: auxin-*a*, 3-indoleacetic acid, pimelic acid, pyruvic acid, uracil, and combinations of nicotinamide and thiamin. The relationship of this substance from liver to those previously described and to the ether-extractable substance for propionic acid bacteria is not clear at the present time.

Butyl alcohol bacteria. Recently Brown, Wood and Werkman

(9) obtained an acidic, ether-soluble fraction from yeast extract which was essential for vigorous growth of butyl alcohol organisms in a medium consisting of hydrolyzed casein, tryptophane, ammonium sulphate, glucose and inorganic salts. When a mixture of 18 purified amino acids was substituted for the hydrolyzed casein the organisms refused to grow. The hydrolyzed casein apparently contained a second unknown factor or else an essential amino acid in addition to those used. Here again, as in much of the previous work reviewed in this section, it appears that at least two substances are needed, one occurring in the acid-ether extract of yeast and the other in hydrolyzed casein. Werkman and associates (9) state that the latter is not thiamin.

Insofar as the requirements have been elucidated, it is evident that these fermentative bacteria, which are not associated with invasion of animal tissues, nevertheless require some of the vitamins which are essential for the higher animal.

Nitrogen-fixing bacteria

Growth of the various types of *Rhizobium* in synthetic media is usually negligible if pure, ordinary ingredients are used. Upon the addition of small amounts of yeast extract the cultures develop readily and evidence has been advanced, notably by Allison and Hoover (1, 46), that the effect of the yeast can be attributed to the presence of small amounts of a growth factor. This substance they termed "coenzyme R." It was found to be present especially in yeast, cane molasses, natural humic acid, commercial egg albumin, and commercial sucrose. It could be obtained along with impurities by extraction of commercial sucrose or dried cane molasses with absolute alcohol. Small amounts of such extracts, when added to the usual synthetic medium, led to good development of cultures of the root-nodule organisms (1). The extracts also stimulated the rate of respiration as determined in the Warburg apparatus.

Hoover and Allison (46) obtained apparently the same factor in more concentrated form from *Azotobacter* cultures which had evidently synthesized it. Attempts to obtain it in crystalline form were not successful. The substance was dialyzable and

quite heat-stable. It was not identical with Williams' pantothenic acid. Cystine and related reducing substances, inositol, synthetic iron humates, and various nucleotides could not be substituted for it. They also report (2) that the growth response of the nodule bacteria to natural humic acid is due almost wholly to this factor and not to the available iron content. The presence of a somewhat similar substance in brown sugar and in calcium sucrate has been noted by Clark (17). This substance was responsible for growth acceleration of *Rhizobium trifolii*. It was destroyed by ashing, was dialyzable, was adsorbed by charcoal and was reported to resemble in some respects the bios complex of yeast.

It is difficult to correlate these reports and at present no conclusion can be reached concerning the nature of any growth factor which might be needed by *Rhizobium* or other nitrogen-fixing soil organisms.

Streptothrix

Attempts were made by Reader and associates to separate growth-promoting substances for *Streptothrix corallinus* from an enzymic digest of beef. No pure compound was obtained but the active material was stated (116) to be organic, water-soluble, ether-insoluble, dialyzable, stable to alkali in the purest preparations, and not precipitated by neutral or basic lead acetate. It was not identical with vitamin B_1 or B_2 preparations (112), but a similarity in constitution to B_1 was suggested. In later work (117) it was found that mannitol, but not the other alcohols commonly used in bacteriological work, considerably increased the mass of growth when added to the salt-sugar medium together with a growth factor preparation. It was believed that the mannitol acted as a specific source of food rather than as an additive growth-promoting factor, and a similar interpretation was suggested for the effect of *i*-inositol (bios I) upon yeast.

The relation of this streptothrix growth substance to the other bacterial growth-promoting factors is not clear. Repetition of the tests with pure preparations of thiamin and the other compounds now available would be of interest.

YEASTS

A consideration of growth accessory substances for the yeasts revolves largely about the question of "bios," the term first applied in 1901 by Wildiers (166) to designate material in yeast extract which was needed for normal cell proliferation of *Saccharomyces cerevisiae* in a synthetic medium. Wildiers and a little later Devloo (22) recorded some of the physical and chemical properties of bios and stated that it was not present in yeast ash. The study of the accessory growth substances for microorganisms may be said to have started with this work.

It will be recalled that up to this time Pasteur's opinion had prevailed, namely that yeast could be cultivated readily in a solution consisting only of sugar, an ammonium salt and ash of yeast. To this Liebig had objected and offered evidence to the contrary, though the weight of opinion continued to favor Pasteur's view. Wildiers proposed a possible explanation for these differences based on size of the inoculum and consequent carrying over of bios. He evidently considered his term bios to be only a tentative one, expressing the hope that a chemical name might later replace it. In this he appears to have been years ahead of his time for a quarter of a century was to elapse before any real progress was made in this direction.

Wildiers' ideas were soon challenged and for a few years a controversy ensued concerning his interpretations and methods. This has been reviewed by Tanner (149) and Buchanan and Fulmer (10). The discussion of bios largely disappeared for a time, only to be revived some years later when it was maintained that vitamin B and bios were the same and that the stimulation of yeast growth by bios supplied a quantitative method of assaying vitamin B (168). It was soon shown that this assumption in its original form was incorrect. However, a renewed impetus was given and much of our present knowledge of bios is due to the intensive studies started at this time by several groups of investigators, especially those associated with Fulmer, Miller, and Williams.

Evidence shortly appeared to show that bios was in reality a complex of a number of substances and that the combination of

all of them was often necessary to exert the stimulating effect (72, 39, 173). Similar situations have been encountered more recently in the study of other growth factors. Along with recognition of the multiple nature of bios further complications arose when it was realized that different yeasts possessed quite different nutritive requirements. In the earlier work there had been a tendency to regard yeast as a single entity. Some of the contradictory statements found in the literature undoubtedly were due to this failure to recognize the marked differences between species and strains. This situation was remedied, however, as attention was directed to the differing nutritive requirements of various yeasts. Lucas (72) found that different strains of yeast varied in their response to bios, and similar findings were reported by Williams, Wilson and von der Abe (173), Copping (18) and others. In the more recent studies this difference in requirements has been well recognized.

Aside from the yeast cell itself, many other sources of bios-like growth stimulants have been reported. Wildiers (166) originally called attention to several of these sources, and more recently the presence of growth factors for yeast has been noted in alfalfa (39), the buds and leaves of a number of plants (19), oat coleoptiles (27), tomato juice (28, 88) and commercial sugars (43). All the evidence indicates a widespread distribution in nature.

Regarding the chemistry of the bios complex, the separation accomplished by Lucas (72) seems to have been the basis for much of the subsequent work. He obtained two fractions, bios I and bios II, by treating with alcoholic barium hydroxide. Separately each fraction possessed little activity but when combined the original activity was restored. In subsequent work by Eastcott (25) the active principle of bios I was identified as *i*-inositol.

Later work showed that bios II was not a single entity and several groups of investigators fractionated it by one method or another. Miller, Eastcott and Sparling (89) recognized a bios II A and bios II B. Crude bios II B contains a new constituent provisionally named bios VII (88). It was reported (87)

that II A could be replaced by β-alanine and leucine. Also a bios V which appeared to be necessary for a certain strain of yeast was reported by Farrel (28). The bios V, however, did not increase the crop of *Saccharomyces cerevisiae* or several other common yeasts. Bios V can apparently be replaced by thiamin (88).

In the meantime Williams and Roehm (170) found that thiamin stimulated growth of some yeasts and pointed out that it possessed certain properties in common with one of the components of bios II. In addition, there was evidence of marked growth-promoting activity in another fraction. In further work Williams and associates (169) reported the presence and partial purification of an acidic substance which markedly stimulated growth. This substance was of widespread occurrence in nature and was called "pantothenic acid." Small amounts of pantothenic acid alone, when added to a basal medium, exerted some growth-promoting effect, but the activity was enhanced by addition of *i*-inositol or thiamin or both (Williams and Saunders, 172). Richards (120) has reported that pantothenic acid stimulates yeast growth by shortening the generation time. This was seen particularly when the seed yeast came from older cultures. There was less effect on the crop.

Williams and Rohrman (171) added β-alanine to the list of growth-promoting compounds. When incorporated in a basal medium containing salts, sugar and inositol, 0.08 microgram per cubic centimeter of β-alanine produced growth stimulation of five yeast strains. The addition of aspartic acid to the medium resulted in a still larger yeast crop, while one of the five strains also required thiamin.

Recently Kögl and Tönnis (59) announced the isolation of a substance called "biotin" which was obtained in crystalline form as its methyl ester. This was isolated from what constituted part of the bios II complex (the fraction adsorbed on charcoal). Biotin possesses a marked stimulating effect on yeast growth, a dilution of one part in 4×10^{11} producing a perceptible effect, while one part in 4×10^{10} caused a more distinct stimulation.

In much of the foregoing work various strains of *Saccharomyces*

were used. Schopfer (134, 135) has recently studied the requirements of several of the torulae, *Rhodotorula rubra* and *R. flava.* These yeasts required thiamin for satisfactory development, and maximum growth was obtained with about 0.4 microgram in 25 cc. of synthetic medium. The pyrimidine component of thiamin could substitute for the whole molecule; the thiazole component was practically without effect. Inositol and pantothenic acid or combinations of both produced no growth-promoting effect upon these two species. Further differences in the requirements of different yeast strains with respect to thiamin and its two component ring structures have been reported by Schultz, Atkin and Frey (140).

In an interesting report Sperti, Loofbourow and Dwyer (146) have directed attention to the liberation by injured cells of substances affecting growth. Cells of *Saccharomyces cerevisiae* injured by ultraviolet irradiation produced substances which stimulated cell proliferation. Apparently the effect was due not merely to substances found in normal cells but to products elaborated by the injured, living, cells as a definite response to injury. These results are of interest in relation to similar evidence concerning the proliferation of cells in tissue cultures. Also they may have a general bearing upon the preservation of communities of microorganisms following injury to some of the cells of the community. Norris and Kreke (105) have presented evidence to show that the factors affecting growth, fermentation and respiration of *Saccharomyces cerevisiae* are not one and the same substance. Using malt combings as a source of bios, they showed that factors which affect these different cell activities could be concentrated in different fractions.

To sum up the evidence, it is quite apparent that the yeasts as a group vary as widely in their requirements of accessory growth substances as do the bacteria. Some yeasts can develop through continuous transplants in simple synthetic media and evidently are able to synthesize all needed compounds. Others are stimulated in greater or less degree by additions of *i*-inositol, thiamin, β-alanine, "biotin" and "pantothenic acid," depending upon their inability to obtain by synthetic or other processes one or more of

these materials. Doubtless still other compounds will be found to be needed by some of the more exacting species.

MOLDS AND HIGHER FUNGI (EUMYCETES)

Although many of the fungi develop readily in very simple solutions containing inorganic nitrogen, a sugar and mineral salts, it is well known that others are more exacting in their requirements and complex mixtures such as peptone, protein hydrolysates and tissue extracts must be supplied for their successful cultivation. Some of them, indeed, are worthy rivals of the more exacting bacteria in their nutritive requirements. As in the case of the bacteria, a number of suggestions were advanced from time to time that vitamin-like substances were needed by these forms (Linossier 71, Willaman 167, Lepeschkin 70). At the time, fifteen to twenty years ago, the evidence for this was necessarily vague. More recently, with increasing knowledge of the vitamins and particularly of the vitamin B complex, this suggestion has been subjected to more direct experimental proof.

Nematospora gossypii was one of the first to be investigated systematically. Farries and Bell (30) reported that it required an "accessory factor" which could be obtained in impure form from egg white, crude casein and other sources. Its exact chemical nature was not determined. This work was confirmed by Buston and Pramanik (16), who separated the factor into two fractions by precipitation with barium hydroxide and alcohol. Neither fraction was active in the absence of the other. The active component of one fraction was identified as *i*-inositol, while the other was concentrated to a considerable degree but not identified (15). In the presence of inositol and a "second accessory factor" from lentils *N. gossypii* grew on a medium whose sole nitrogenous constituent was asparagine or ammonium aspartate (15a). It seems likely that this second fraction contained the biotin of Kögl or a mixture of biotin and thiamin, for Kögl and Fries (58) secured growth of *N. gossypii* in a synthetic medium containing these substances together with *i*-inositol.

Recent studies, particularly those of Schopfer, have emphasized the importance of thiamin for a number of the fungi. Growth

and zygospore formation of *Phycomyces blakesleeanus* (a *Mucor*) took place in a synthetic medium following the addition of crystalline thiamin. In fulfilling the nutrient requirements of this mold, thiamin satisfactorily replaced concentrates prepared from yeast or other sources (129, 12). Riboflavin showed no such effect (12). This work was soon extended to include additional species of molds (130, 131) belonging to the genera *Absidia, Parasitella, Mucor, Pilaria* and others, many of which were found also to require thiamin. In contrast to these results *Rhizopus* was inhibited. Results obtained with natural thiamin were shortly confirmed by the use of a synthetic preparation, and maximum growth of *Phycomyces* was secured upon the addition of 0.5 microgram to 25 cc. of medium (132).

In the investigation of other sources of growth-promoting substances, Schopfer (131) reported that wheat-germ extract apparently contained at least one other factor in addition to thiamin, for small amounts of the extract produced rapid development of *Rhizopus*. He also found (133) that extracts of the leaves of many species of higher plants supplied substances producing a similar effect on *Phycomyces*. This material could be extracted with alcohol, was thermostable in acid solution and was adsorbed by fuller's earth and animal charcoal. In a further study of wheat-germ, Schopfer and Moser (139) described procedures for separation and concentration of several factors showing activity for both *Phycomyces* and *Rhizopus*, especially two factors which they designated "MR" and "MP." Thermostability, resistance to alkali and adsorption by animal charcoal were useful for differentiation. It was suggested that MP might be a disintegration product of thiamin. Certain other preparations or definite compounds could not replace the wheat-germ factors. Thus, pantothenic acid, either alone or with *i*-inositol, was without effect on *Phycomyces* and furthermore it did not augment the action of thiamin. Also heteroauxin (3-indoleacetic acid) had no effect on *Phycomyces* or *Rhizopus*.

Nielsen and Hartelius (103) reported that *Rhizopus* cultures produce a substance, "Wuchsstoff B," stimulating the growth of *Aspergillus*. A stimulant in beer wort for *A. niger* and for yeasts

was later subdivided into several components (104). Certain of these stimulants were required for yeasts and were easily oxidized while others affected mold development and were more resistant to oxidation by hydrogen peroxide and potassium permanganate. The presence of metals (co-growth substances) was also emphasized by Nielsen (102). The influence of growth factors upon development and nitrogen assimilation of *A. niger* was studied by Bünning (11). Thiamin as a rule exerted little growth-promoting effect, while riboflavin led to an increase of about 30 to 40 per cent in the dry weight of the mycelium, though amounts of about 4 to 20 micrograms per cubic centimeter were necessary to bring about this effect. Both of the vitamins as well as several unknown growth factors promoted absorption of nitrates by the mold and this in turn was connected with an intensified respiration.

Mosher, Williams and associates (90) studied the nutritional requirements of *Trichophyton interdigitale*. In addition to rather specialized requirements with respect to inorganic ions and amino acids, this fungus apparently requires at least four accessory substances for satisfactory development. These are: thiamin, riboflavin, *i*-inositol and Williams' pantothenic acid.

A study of the requirements of a number of different fungi, including representatives of the Phycomycetes, Ascomycetes and Basidiomycetes was made by Kögl and Fries (58). Biotin, thiamin and *i*-inositol were tested in a basal medium of glucose, tartrate, and inorganic salts. The thiamin requirement of *Phycomyces* was confirmed. In addition a number of species of the Ascomycetes and Basidiomycetes were found either to require thiamin or to be stimulated by it. A few exceptions to this requirement were also encountered, thus *Nematospora gossypii* required biotin and *i*-inositol for appreciable growth which was further increased by addition of thiamin, while *Lophodermium pinastri* needed biotin and thiamin. β-Alanine was without effect on these types. Kögl and Fries believed that in those cases where a particular factor was found to be unnecessary it was synthesized by the mold. Pairs of fungi with complementary requirements could be grown on a medium without any of the

three factors, although growth was slower under these conditions.

Several recent reports have dealt with the effect on molds of the components of the thiamin molecule. The need for the pyrimidine and thiazole components appears to differ with various molds. In the study of *Phycomyces*, Schopfer and Jung (138) reported that each of these components alone had little or no effect but in combination the effect was identical with that of the whole thiamin molecule. Similar results were reported by Sinclair (143) who found also that thiamin diphosphate (cocarboxylase) was about as active as the vitamin itself. Robbins and Kavanagh (124) found that a mixture of the thiazole component with a 5-bromomethyl derivative of the usual pyrimidine was as effective as molar equivalent amounts of thiamin. The vitamin was therefore believed to be synthesized by the mold from the separate components, since they were required in molecularly equivalent quantities. Additional data on the effectiveness of derivatives of thiamin and its two components with respect to microorganisms in general will be discussed in a later section.

Thus the impure fractions from plant and animal tissues can be replaced in a few instances by small amounts of definite chemical substances. Of the compounds thus far demonstrated to possess growth-promoting activity for the fungi, thiamin assumes an important part, as it does with the bacteria and higher forms of life. Presumably many of the parasitic fungi are unable to synthesize this molecule or one of its components and so fail to develop. Also, there is evidence of the need for a number of interacting factors, the absence of any one of which may lead to complete failure or a marked retardation in development. Inositol seems to be needed in some instances; likewise the preparations known as biotin and pantothenic acid. Future work will doubtless supply further evidence concerning the nature of these substances and bring to light still others now unrecognized.

MISCELLANEOUS STUDIES

In addition to the work on groups of bacteria treated in the preceding sections, several other studies may be mentioned here.

From peptone and from blood, Sahyun, Beard and associates (125) obtained in partially purified form "activators" which stimulated cell multiplication of *Escherichia coli*. This effect was in addition to that exerted by known amino acids, and the activating substance was not destroyed by growth of the organisms in media containing it. Dunn and Salle (24) extracted stimulating agents from rice bran with 60 per cent methanol and 25 per cent ethanol. Evidently the rice bran extract also contained food material and inorganic salts. The growth of carbohydrate-fermenting organisms was greatly enhanced and it was suggested that the stimulating agent might be carbohydrate in nature, but was not glucose.

Koser, Chinn and Saunders (60) found that certain gelatins contain growth factors for many of the commoner pathogens, including such types as hemolytic streptococci from scarlet fever, pneumococci, *Brucella* and others. In a synthetic medium, in which these organisms were unable to develop, the addition of some gelatins promoted ready growth of these types. A more highly purified photographic gelatin did not support growth under the same conditions.

Protozoa. While no attempt has been made to review exhaustively the literature dealing with the protozoa, several instances may be cited to show the importance of the accessory growth factors for development of certain of these forms. M. Lwoff and A. Lwoff (80, 73) found that hematin, protohemin, and protoporphyrin could replace an essential substance supplied by blood for cultivation of several trypanosomes of the genera *Strigomonas* and *Leptomonas*. Since protoporphyrin contains no iron the trypanosomes can evidently combine this molecule with traces of iron present in the medium and thus construct the iron-containing hematin. Lwoff and Dusi (74) and Lwoff and Lwoff (82, 75) have shown that a number of different forms (*Polytomella caeca, Polytoma caudatum, P. ocellatum, Chilomonas paramecium, Glaucoma piriformis* and *Strigomonas oncopelti*) need thiamin. In addition one or more other factors are probably required by some of these types. According to a recent report (83) *Schizotrypanum cruzi* requires ascorbic acid and hematin.

THE ROLE OF INORGANIC SALTS IN PROVIDING GROWTH-PROMOTING EFFECTS

One explanation of the growth-promoting properties of tissue extracts is based on the assumption that the effect may be due to the presence of certain inorganic salts, which are needed by the microorganism. Of the enormous literature dealing with the effects of inorganic salts upon microorganisms, the following may be cited as bearing more particularly upon our subject. Webster and Baudisch (163) and Baudisch (4) stressed the importance of certain "active" forms of iron salts and iron oxides which might function as the X factor in the growth of hemophilic bacteria. Reed and Rice (118) secured heavier growth of the tubercle bacillus and of several related acid-fast types in a synthetic medium when small amounts of iron and citrate were added. The citrate prevented precipitation of the iron. Elvehjem (26) emphasized the importance of iron and copper in the growth and metabolism of yeast and suggested that a considerable part of the beneficial action of bios depended upon changes which made iron more available for assimilation. Greaves, ZoBell and Greaves (42) reported that growth of yeasts in a mineral salt-sugar solution was increased by minute amounts of iodine. Richards (119) stressed the importance of thallium and expressed the belief that this element may be one of the growth stimulants for yeast that have been referred to as bios. Thallium in varying amounts was present as an impurity in different brands of asparagine.

Burk, Lineweaver and Horner (13) reported that growth stimulation of *Azotobacter* by humic acid was due to the iron content of the latter and that natural humic acid could be replaced by several organic or inorganic iron compounds. Thorne and Walker (156) found that growth of several species of *Rhizobium* in a purified sucrose-nitrate medium was greatly increased by the addition of small amounts of iron, especially ferric chloride. The importance of molybdenum and zinc for development of *Aspergillus niger* was emphasized by Steinberg (147). The decreased yield of mold growth obtained when purified sucrose was used in a synthetic medium was interpreted as being due to the removal of small amounts of molybdenum and zinc from the

sucrose, rather than to the removal of bios or other accessory growth substances.

These references and others of a similar nature present an impressive argument for the inorganic salts, and it is not surprising that a number of the foregoing workers expressed doubts of the existence of accessory growth substances of organic nature in yeast decoctions or tissue extracts.

On the other hand, there is evidence that ashing of tissue preparations destroyed the growth-promoting effect. The writers (66, 126) found that ashing of active fractions obtained from veal infusion, liver, spleen, yeast and white potatoes caused a complete loss of the growth-promoting property. Schopfer and Moser (139) in studying the factors in wheat germ for molds state that the mineral substance present in the ash of several extracts was not responsible for the growth-promoting effect. Tatum, Peterson and Fred (150) ashed the Neuberg filtrate fraction in connection with their work on propionic acid bacteria and found that the ash did not produce the stimulative effect of the original extract. Clark (17) found that ashing and wet combustion destroyed the growth factor for *Rhizobium*. M. Lwoff (81) reported that "active" iron compounds, as employed by Baudisch for *H. influenzae*, were not effective as substitutes for hemin in supplying the needs of the trypanosome *Strigomonas fasciculata*. The writers and their associates (62) were unable to demonstrate any growth-promoting effect when various amounts and combinations of inorganic salts, particularly those of the heavy metals, were substituted for active growth-factor preparations from tissues.

Concerning the importance of the inorganic salts and particularly of the metals which act as catalysts in biological systems there can be no doubt whatsoever. It is unfortunate that our knowledge of the mineral requirements of microorganisms is so incomplete that we are continually uncertain, when attempting cultivation in simplified media, whether the proper compounds or the proper amounts have been supplied. However, this objection has been met by some workers who have employed the ash of biological materials which support growth.

It has been common practice to ignore the traces (or perhaps larger amounts) of these compounds which are present as impurities with the amino acids, sugars and other ingredients used for synthetic media. Glassware, metallic filters and other sources contribute an additional supply.

Doubtless, if we knew more of the mineral requirements of the microorganisms our efforts to obtain satisfactory and rapid growth of the fastidious types in synthetic media would be more successful. Aside from these important inorganic ingredients, however, recent work has revealed the significance of organic entities which are essential for the development of some of the more exacting bacteria, yeasts and molds. It would appear, therefore, that the basic idea of searching for such organic compounds need not be altered, but that along with such endeavor there should be an alert recognition of the importance of the inorganic constituents.

GROWTH-PROMOTING EFFECTS AND REMOVAL OF INHIBITING AGENCIES

Another explanation for the growth-promoting effects which follow the addition of tissue extracts to a simplified medium is that the added organic matter has combined with certain "toxic" or inhibitory substances present in the medium, thereby removing a harmful agent which previously restrained cell proliferation. This suggestion was advanced by Fernbach (31) and Windisch (174) in the early discussions on the effect of bios on yeast growth, and it has since appeared from time to time in connection with the studies on bacteria. Windisch in particular called attention to the presence of copper in distilled water and in media.

It seems unnecessary to review here the many reports dealing with possible inhibitory effects of the varied components of culture media. One example, taken from the more recent literature, will serve as illustration. O'Meara and Macsween (106, 107) found some commercial peptones contained sufficient copper to inhibit growth in ordinary nutrient broth when the inoculum consisted of only small numbers of cells. The addition of blood serum to the medium rendered it suitable for growth, presumably

by combining with or precipitating the copper. Here is an excellent example of apparent growth-promoting or growth-stimulating effect following the addition of blood serum. While the possibility of such effects must always be kept in mind, there now appears to be ample evidence that growth factor activity cannot be accounted for solely on this basis.

GROWTH-PROMOTING EFFECTS RESULTING FROM CHANGES IN PHYSICAL PROPERTIES OF THE MEDIUM

In the attempts to develop suitable culture media for the more exacting microorganisms there is evidence that the physical character of a medium is not only important, but at times may be *the* factor determining suitability of the medium. With respect to the study of growth-promoting factors, various investigators have attributed the beneficial effect of tissue extracts to changes produced in the physical character of the medium.

Differences in hydrogen-ion concentration, surface tension, osmotic pressure, and the oxidation-reduction potential are among the more obvious alterations which may result from the addition of tissue extracts or other growth-factor preparations. Of these various properties the importance of a suitable pH is well recognized, and the oxidation-reduction potentials of culture media and of developing cultures have received serious study. Less attention has been paid to the other properties. Since several publications have emphasized particularly the possible misinterpretations of growth-factor effects due to changes in the oxidation-reduction potentials of the culture medium, most of our discussion here will be concerned with this aspect of the problem, but it must be realized that the same principles apply to the other physical properties.

There is now considerable evidence that bacteria can multiply only in media where the redox potential is within certain limits and that the limiting zone, whether broad or narrow, varies with the individual organisms. The favorable conditions for growth which are brought about by various procedures, such as the addition of tissue extracts, large inocula, boiling of the medium, etc., have been attributed, at least in part, to the reduction of oxidized

substances or to the establishment of a suitable reduction potential in the medium.

Wright (177, 178) called attention to inhibitory properties of the usual peptone-infusion media, particularly when seeded with small numbers of cells, and attributed this effect to constituents of peptone in the oxidized state. Heating the peptone solution with meat, during the course of preparation of the medium, improved its growth-promoting properties, and Wright believed this effect was due to reduction of the peptone, or certain of its constituents, thereby removing the toxic action. He also suggested that the inhibitory effect must be taken into account in experiments relating to accessory growth factors. Dubos (23) reported the presence in peptone of substances which were bacteriostatic in the oxidized state. Their bacteriostatic action could be overcome by the addition of thioglycollic acid.

Allyn and Baldwin (3) have also emphasized the importance of the oxidation-reduction character of media in the initiation of growth. A yeast-mannitol medium supported growth of *Rhizobium* when inoculated with small numbers of cells, while in a nitrate-mannitol medium no growth occurred unless very large inocula were used. The yeast medium was more reducing in nature than the nitrate-mannitol medium. The nitrate-mannitol medium permitted growth with similar small inocula after the addition of thioglycollic acid, powdered agar, or other reducing agents. In this instance a synthetic medium, upon the addition of reducing agents, supported growth as readily as a yeast medium. Thorne and Walker (156) found that the addition of reducing agents such as cysteine or thioglycollic acid increased growth and oxygen utilization of *Rhizobium* in media composed of highly purified ingredients (nitrate, sucrose, and inorganic salts). Cysteine brought about increases comparable to those induced by brown sugar, which has been said to contain appreciable quantities of accessory factors. They found no evidence that root nodule bacteria require any complex, unidentified substances for their growth. From these reports it is evident that a growth-promoting effect may be the result of adjustment of the oxidation-reduction potential from a less to a more favorable

region, or from the reduction of oxidized ("toxic") substances in the medium.

The importance of CO_2 tension in the cultivation of bacteria has been stressed by many workers and has been well reviewed by Knight (52). The effect of other changes in the physical character of the medium has also been reported. Hitchens (44) recommended the addition of 0.1 per cent agar to ordinary broth. In the resulting semi-solid medium a number of the more fastidious types developed more luxuriantly than in broth or on ordinary solid agar slants. Another interesting example has appeared in studies on methane fermentation. Breden and Buswell (8) found that addition of shredded asbestos to a liquid medium provided a suitable background for development of the methane-producing types which appeared to require the presence of finely-divided material in suspension. With the shredded asbestos in place of sewage sludge, subcultures could be carried through many transplants.

While it is true that many of the studies on growth-promoting substances have ignored possible changes in oxidation-reduction potential and other physical characteristics of the medium, the growth-promoting effects observed probably are not due to physical changes. In the study of *Lactobacillus delbrückii*, Snell, Tatum and Peterson (145) noted that the addition of potato extract lowered the oxidation-reduction potential of the basal medium and produced a growth-stimulating effect. However, substitution for the potato extract of agents such as cysteine, cystine or thioglycollic acid, which lowered the potential in like amount, did not produce the stimulating effect. Rahn and Hegarty (114) found that substances used to lower the redox potential failed to stimulate and at times even slightly retarded acid production by *Streptococcus lactis*. Koser, Saunders and associates (62) found that changes in the physical properties of the test medium suggested by the foregoing reports did not produce the growth-promoting effect shown by extracts prepared from tissues.

In studying *Streptothrix corallinus* in a synthetic medium plus tissue concentrates, Reader (115) found that alterations of the

surface tension of the fluid, within ordinary limits, did not affect the amount of growth and concluded that the growth-promoting activity of the added concentrates was not due to lowering of tension of the medium.

Our evaluation of these conflicting viewpoints leads to a conclusion similar to that expressed in the previous discussion on the effects of inorganic salts. There can be no doubt of the importance of the physical character of the medium. Unfavorable levels of redox potential or other less well-recognized properties may prevent cell proliferation as effectively as unfavorable ranges of hydrogen-ion concentration. In some cases an apparent growth-promoting effect may well have been due to the alteration of such conditions. Unfortunately, we are still quite vague as to what many of the physical specifications should be and so the whole subject is left in a rather uncertain state. It seems doubtful, however, that the growth-promoting effects of minute amounts of such compounds as thiamin, nicotinic acid, and β-alanine can be explained as due to a change in the physical properties of the medium.

DEFINITE COMPOUNDS WHICH SHOW GROWTH-PROMOTING ACTIVITY

By way of summary, the compounds which have been substituted successfully for the complex mixtures of plant and animal tissue extracts are listed in table 1. Only those substances which seem to fill a fundamental and often specific need for cell proliferation are included. With one exception, the chemical structure of all of these compounds is now definitely known.

Hematin or hemin. This iron-containing compound needs little comment here since it has been discussed in earlier reports. It appears to have been the first of the so-called accessory substances for microorganisms to be definitely identified. In addition to its important rôle in the cultivation of bacteria, it has also been shown to substitute for a component of blood in the cultivation of several trypanosomes.

i-Inositol. The inclusion of *i*-inositol in our list may be open to some question since by itself it is not sufficient for cell multipli-

cation, but the presence of one or more "cofactors" is necessary. Furthermore it usually must be supplied in larger amounts than

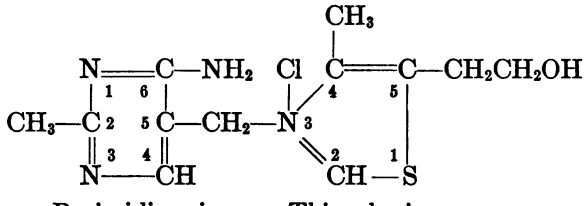
TABLE 1

Compounds which show growth-promoting activity

SUBSTANCE	ORGANISM	COFACTOR	REFERENCE
Hemin	*Hemophilus influenzae*	"V" factor	(20, 21, 153, 154, 155, 32)
i-Inositol	*Saccharomyces*	Other unknown substances	(25)
i-Inositol	*Nematospora gossypii*	Other unknown substances	(16, 58)
Thiamin	Molds	Other unknown substances	(129, 12, 130, 131, 58)
Thiamin	Propionic acid bacteria	Ether sol. factor from yeast	(152)
Thiamin	Yeasts	*i*-Inositol	(172)
		"Pantothenic acid"	(170, 171)
		"Biotin"	(59)
Thiamin	*Staph. aureus*	Nicotinic acid	(53)
		Nicotinamid	
Riboflavin	Lactic acid bacteria	Hydrolyzed casein	(175, 108)
		Ether sol. factor from yeast	
Nicotinic acid and derivatives	*Staph. aureus*	Thiamin	(53, 68)
Nicotinic acid and derivatives	*C. diphtheriae*	β-Alanine	(95)
Nicotinic acid and derivatives	*Shigella paradysenteriae*	None	(61)
Cozymase	*Hemophilus parainfluenzae*		(76)
β-Alanine	*Saccharomyces*	Aspartic acid	(171, 87)
		Inositol	
		"Pantothenic acid"	
		Thiamin	
		Leucine	
β-Alanine	*C. diphtheriae*	Nicotinic acid	(97, 63)

the other growth factors. It has been included, nevertheless, because it represents one of the few instances where a definite compound has been identified as the active ingredient of a growth-

promoting preparation. Its function in cell metabolism seems uncertain at the present time. According to Eastcott (25) it is stored in the cells, since the inositol taken up by yeast from the culture medium can be quantitatively recovered by hydrolyzing the yeast crop.



Thiamin

Thiamin. Studies of the two ring structures which compose the thiamin molecule have revealed an interesting diversity of requirements among those microorganisms for which this substance is effective as a growth factor. In a few instances the *intact* thiamin molecule is required. The pyrimidine and thiazole components when supplied as separate entities, in equivalent molar concentrations, are ineffective as a substitute for the whole molecule. The two components cannot substitute for thiamin in the case of the protozoa *Strigomonas oncopelti* and *Glaucoma piriformis* (82, 75). A similar need for the intact thiamin molecule has been reported for certain of the parasitic fungi, namely several species of *Phytophthora* (123a) and the basidiomycete *Ustilago scabiosae* (136). For the related *U. violacea* thiamin can be replaced partially by the two components. Evidently these microorganisms are unable to put together the two components to form the whole thiamin molecule or, in the case of *U. violacea*, this synthesis is accomplished too slowly to permit normal development.

Other types are somewhat less exacting in their requirements. *Phycomyces blakesleeanus* requires both components of the thiamin molecule but not the intact molecule itself (138, 143, 124). This is also true of *P. nitens* (124a), *Staphylococcus aureus* (54) and the flagellate protozoan *Polytomella caeca* (74). It appears that these

microorganisms are not able to synthesize either of the two component ring structures. The molds *Absidia ramosa* and *Parasitella simplex* require both components for rapid development but can grow more slowly in the presence of the pyrimidine constituent alone (134). Apparently the thiazole is synthesized by these molds, but in an amount insufficient for normal growth.

Still other microorganisms can develop as readily in the presence of only one of the components as when the whole thiamin molecule is supplied. This is true of *Mucor ramannianus* (100) which needs only the thiazole constituent and also of the yeast, *Rhodotorula rubra* (135) and several higher fungi which require only the pyrimidine constituent (124a). There is some evidence that the component which is not required is synthesized by the organisms.

In contrast to the foregoing are the many microorganisms which are able to develop in a synthetic medium devoid of thiamin. While our knowledge of the physiology of these types is still quite incomplete, it appears probable that thiamin plays an important rôle in their metabolic processes. Since in these cases neither thiamin nor its direct components are supplied, these organisms apparently possess the property of synthesizing the two ring structures from much simpler compounds.

Derivatives of thiamin and its components. There is evidence of a high degree of specificity in the chemical structure of the active compounds. Thiochrome, an oxidation product of thiamin in which the nitrogen atom of the 6-amino group of the pyrimidine is linked to the 2-carbon atom of the thiazole ring, can substitute for thiamin only very imperfectly or not at all for growth of *Staphylococcus aureus* (54), *Phycomyces* (137) and *Rhodotorula rubra* (134). Also, a molecule similar to thiamin but lacking the β-hydroxyethyl group at the 5-position of the thiazole ring was inactive for *Staphylococcus* (54). Substitutions in various positions of the pyrimidine ring of the intact thiamin molecule greatly reduced or abolished the activity of thiamin (55a). The activity could be restored by addition of the normally substituted pyrimidine.

Several substitution products of both the pyrimidine and the

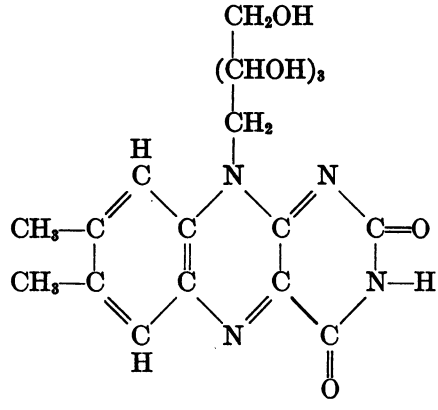
thiazole components have also been tested. 2-Methyl-5-aminomethyl-6-aminopyrimidine[2] was active for *Staph. aureus* in the presence of the thiazole component, while under the same conditions 2-methyl-5-hydroxymethyl-6-hydroxypyrimidine, 2-methyl-5-aminomethyl-6-hydroxypyrimidine and 2-hydroxy-4-aminopyrimidine (cystosine) were all inactive (54). When the 5-aminomethyl group in 2-methyl-5-aminomethyl-6-aminopyrimidine was replaced by a 5-thioformamidomethyl group, the compound retained activity, though in somewhat lessened degree, for *Staphylococcus* (54) and for *Phycomyces* (143) and was said to "substitute fully" for growth of *Rhodotorula rubra* (134). Upon substitution of a 5-bromomethyl for the 5-aminomethyl group, growth-promoting activity was retained for *Phycomyces* (in the presence of the thiazole component) (124). In a later report Knight and McIlwain (55a) used additional substituted pyrimidines and found that most of them were inactive for *Staph. aureus*. The groups attached to the ring which appear essential for activity are: a methyl group at position 2, an amino group at position 6 and a methyl group substituted in certain ways at position 5. Thus at position 5, $-CH_2NH_2$, $-CH_2OH$ and $-CH_2NH \cdot CSH$ permitted growth, but $-CH_3$ and $-CH_2CO \cdot NH_2$ were inactive. Nucleic acid or hydrolysates of nucleic acid which supply pyrimidines were not effective for *Phycomyces* when substituted for the specific pyrimidine (124).

Substitutions in the thiazole component have demonstrated a similar high degree of specificity. For growth of *Mucor ramannianus* 4-methyl thiazole, 4,5-dimethyl thiazole and 2-mercapto-4-methyl thiazole were all unable to take the place of the usual 4-methyl-5-hydroxyethyl thiazole (100). For growth of *Phycomyces*, Robbins and Kavanagh (124) found that a number of other thiazole derivatives were ineffective as substitutes for the usual component. Likewise a number of sulphur-containing compounds such as methionine, glutathione, thioglycollic acid and others were ineffective. For growth of *Staph. aureus* Knight

[2] The designation of the pyrimidine derivatives has been changed in this article to conform with the usual system of numbering the positions in the pyrimidine ring.

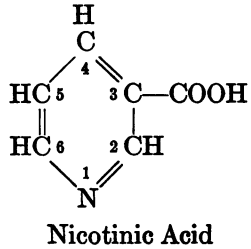
and McIlwain (55a) found other substituted thiazoles were either inactive or showed reduced activity.

Judging from the relative effects on *Staph. aureus* of the different substituted groups at position 5 of the pyrimidine, it appears probable that the pyrimidine and thiazole components are joined to form the intact thiamin molecule, rather than that the two components are used separately (55a). Certain observations of Hills (43a) on pyruvate metabolism by *Staph. aureus* support this hypothesis, as does the work of Robbins and Kavanagh with *Phycomyces* (124).

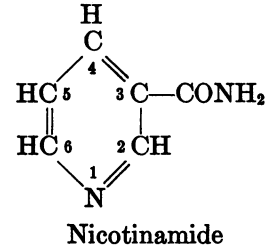


Riboflavin

Riboflavin. Little information concerning riboflavin appears to be available aside from that given in the references previously listed. Other related compounds possessing the isoalloxazine or alloxazine rings have not been available or their activity has not been tested in bacteriological work.



Nicotinic Acid



Nicotinamide

Nicotinic acid and nicotinamide; coenzyme; cozymase. The recent work of Warburg and of Euler and their associates demonstrated that nicotinamide is a constituent of the coenzyme[3] from horse blood and the chemically related cozymase[3] from yeast. The nitrogen in the pyridine ring of nicotinamide is important in the transfer of hydrogen in biological oxidations. It is of interest that in the case of *H. parainfluenzae* (76), the pyridine nucleotide di- or tri-phosphate was required and nicotinic acid, nicotinamide, and adenylic acid (adenine + d-ribose + phosphoric acid) could not substitute as growth factors. In the case of the staphylococcus, the diphtheria bacillus and the dysentery bacilli only the nicotinic acid or its amide was needed.

The comparative activity of nicotinic acid, nicotinamide and some related compounds has been studied in a few cases. According to Mueller (95) the amide was about one-tenth as effective as the acid for the diphtheria bacillus. In studies on the staphylococcus Knight (53) found the amide to be about five times more potent than nicotinic acid in the presence of appropriate amounts of thiamin. Methyl nicotinate also was effective but development of cultures was slower. Pyridine-3-nitrile was ineffective as such but was active after hydrolysis (yielding nicotinic acid). In a later publication Knight and McIlwain (55a) reported that the following compounds were all inactive: coramine, pyridine-3-sulfonic acid, β-picoline, nicotine, trigonelline methyl sulphate, trigonelline chloride, isonicotinic acid, picolinic acid, quinolinic acid, 2,4-dimethylpyridine-3,5-dicarboxylic acid and 2,4,6-trimethylpyridine-3,5-dicarboxylic acid. Landy (68) has reported that the two isomers of nicotinic acid (picolinic and isonicotinic acids) cannot replace nicotinic acid for the growth of *Staph. aureus;* α- and γ-picoline were also ineffective. Nicotinamide and the N-ethyl nicotinamide were both active but the N-diethyl compound was inactive. Sodium and ammonium nicotinate were as effective as nicotinic acid itself, but the ethyl ester of the acid was slightly less active.

In studies on the dysentery bacilli Koser, Dorfman and Saun-

[3] Constituents of coenzyme and cozymase are adenine, nicotinamide, 2 molecules pentose and either 3 or 2 molecules, respectively, of phosphoric acid.

ders (61) found the amide to be slightly more effective, but the difference between the amide and the acid was not marked. In a more extended study of nicotinic acid derivatives Dorfman, Koser and Saunders (22a) showed that pyridine-3-sulfonic acid, trigonelline, 6-methyl-nicotinic acid, nipecotic acid, isonicotinic acid, β-acetylpyridine, β-picoline, and pyridine were devoid of growth-promoting activity. The following substances favored good growth in the dilutions indicated: nicotinic acid, nicotinamide, methyl nicotinate M $\times 10^{-7}$; trigonelline amide, ethyl nicotinate, nicotinuric acid, ethyl nicotino-acetate M $\times 10^{-6}$; nicotinic acid N-methyl amide M $\times 10^{-5}$; nicotinonitrile M $\times 10^{-4}$. Picolinic acid and quinolinic acid showed activity at a dilution of M $\times 10^{-4}$ but there is a possibility that these two preparations may have been contaminated with traces of nicotinic acid.

Beta-alanine. The growth-promoting activity of this amino acid for some yeasts and the diphtheria bacillus is quite in contrast to the negative results secured with the ordinary α-alanine. Many of the protein hydrolysates or basal synthetic media in which the diphtheria bacillus has failed to develop have contained α-alanine. Addition of one microgram or less of β-alanine per cubic centimeter of medium fills some need for cell multiplication which is not supplied by the α-form. This importance of a β-amino acid is of particular interest since in the past biologists and chemists have considered the α-amino acids as being the only ones of biological importance. In view of the incomplete knowledge of the composition of proteins and other tissue extractives, it may well be that β-amino acids play a far more important rôle than has heretofore been recognized.

The diphtheria bacillus is capable of obtaining β-alanine from naturally occurring *l*-carnosine but not from the *d*-form (Mueller, 96). Upon acid hydrolysis, both *d*- and *l*-carnosine yield equally active products.

It might also be added that asparagine and aspartic acid which have been commonly used in synthetic media, can yield β-alanine and it is quite possible that many organisms capable of developing in the simpler synthetic media can bring about this change and

secure β-alanine from asparagine. In other words, the need for β-alanine may be much more wide-spread among microorganisms in general than indicated by the results with some yeasts and the diphtheria bacillus, but many types may secure it from asparagine or other sources.

Biotin. Tentative empirical formula, $C_{11}H_{18}O_3N_2S$. This substance has been included in the list of growth factors although its structural formula is not yet known. From the reports of Kögl and associates (57) it appears to be a definite entity. It is an amphoteric substance and its methyl ester has been obtained in crystalline form. The evidence thus far submitted seems to show that it is important in the cultivation of a number of microorganisms belonging to quite different groups and that very minute amounts of it exert a distinct growth-promoting effect.

SYNTHESIS OF ACCESSORY GROWTH FACTORS. MUTUAL INFLUENCES

It seems reasonable to believe that many of the growth factors listed in the foregoing section and others still unrecognized are required by microorganisms in general. For many types there is no need to supply them as such, because the organisms presumably can synthesize them from simpler substances. Here and there, however, we encounter a type which is unable to synthesize *one* of a number of required substances (e.g., dysentery bacilli and nicotinic acid). When this one compound is supplied along with needed sources of nitrogen, energy and inorganic salts, rapid multiplication ensues. Other organisms happen to be unable to manufacture *two* of these substances (e.g., the staphylococcus with respect to thiamin and nicotinic acid) and do not develop unless both are supplied. Either compound in suboptimum amount limits growth. Again, an organism may be totally unable to synthesize one needed compound but can construct another required substance slowly—too slowly for normal growth. Here one substance is essential and another serves to stimulate growth. In a similar way, other more exacting microorganisms will doubtless be found to need an assortment of various substances which they themselves cannot produce. Thus,

there are a number of interacting compounds and often the action of any one becomes evident only in the presence of the others. It is obvious, too, that the building material which is available for the organism will doubtless vary from one situation to another so that the kind of "raw product" offered may often determine in large measure whether or not certain compounds can be synthesized.

It is believed that the lack of synthetic abilities, with resulting "fastidiousness" of the organism, represents a loss of properties in connection with adaptation to a commensal or a parasitic mode of life and that it is not due to the acquisition of new growth requirements (52, 76). In nature those organisms which are unable to accomplish such syntheses must depend upon production of the required compounds by other types. Many of the instances of growth stimulation of one type by another, seen on the ordinary laboratory media, can doubtless be explained on this basis.

The familiar "satellite" phenomenon of Grassberger (41), who called attention to the increased size of colonies of *H. influenzae* when growing in close proximity to colonies of staphylococci, has been often encountered with many other species. A few instances associated with definite growth factors follow. In the work on thiamin Müller and Schopfer (100) found a mold (*Mucor ramannianus*) which was incapable of synthesizing one component of the thiamin molecule and so was unable to develop unless this structure was supplied. A yeast (*Rhodotorula rubra*) needed only the other thiamin component. These organisms were capable of developing together in a simple medium, without any added thiamin, since each manufactured the particular component of the thiamin molecule needed by the other. Another instance was reported by Kögl and Fries (58) with respect to *Polyporus adustus* and *Nematospora gossypii*. Neither of these fungi was able to grow in a synthetic medium in pure culture; when inoculated together, however, they developed. *Polyporus* requires thiamin which was supplied apparently by *Nematospora*, while the biotin requirement of *Nematospora* was supplied by *Polyporus*. With the varied synthetic abilities of diverse organ-

isms and the many situations encountered in nature there would seem to be almost no limit to the number of such combinations.

Other striking relationships have been reported between microorganisms and the higher plants. Of particular interest are those concerning the fungi and orchids, and the relationships between the root-nodule bacteria and legume plants. A review of this aspect of the problem has been given by Bonner (5). A fuller recognition of the limitations of synthetic abilities of organisms would doubtless help in no small degree in explaining some of the baffling symbiotic and other relationships so frequently encountered in nature. The apparent inability of many of the pathogenic microorganisms to synthesize accessory growth factors, such as nicotinic acid and thiamin, seems highly significant in connection with their invasion of the tissues of the higher animals and plants where these substances may be found.

FUNCTION OF THE ACCESSORY GROWTH FACTORS

What essential rôle is played in the physiological processes of microorganisms by the minute amounts of these growth substances? A consideration of the substances now known to possess growth-promoting properties shows that most of them enter into the structure of enzymes or coenzymes concerned with cell oxidations. Thus, the pyrophosphoric ester of thiamin, thiamin diphosphate, functions as a cocarboxylase with a protein of yeast cells and in this enzyme system strongly promotes the decarboxylation of pyruvic acid, an important intermediate product in the dissimilation of glucose. Nicotinamide is one of the components of the coenzyme of Warburg and of cozymase which plays an important rôle as a mediator in biological oxidation. Riboflavin when combined with phosphoric acid and protein becomes the "yellow enzyme" of Warburg and Christian which, together with a second enzyme and the coenzyme, brings about the oxidation of hexose-monophosphoric acid ester, an important step in sugar oxidation.

In these cases, the fulfillment of the "growth factor" requirements of one of the more fastidious microorganisms furnishes a portion of an enzyme or coenzyme molecule which the organism

itself cannot synthesize, but which it needs in order to carry on its metabolic processes. Without the needed component the sequence of events in the respiratory chain is broken and cell multiplication is not possible. Since these substances enter into a catalytic respiration system it becomes apparent why such minute amounts suffice.

A similar function has been suggested for hemin (80, 73, 78) in relation to certain trypanosomes and *H. influenzae*. In the past, much attention has been centered on the peroxidase or catalase activity of X factor as a protective mechanism against toxic peroxides. From the work of the Lwoffs, however, it appears reasonable that the need of the hemophilic microorganisms for hemin or X factor is connected with inability to synthesize the prosthetic group of a respiratory enzyme.

In past years, attempts to cultivate the more exacting types in chemically definite media have considered for the most part only the question of structural material for the cell proteins and neglected the materials required for the building of enzyme systems or other special needs. Rahn (113) has suggested that the vitamin-like substances might be needed for construction of certain special molecules in the cell, for example the genes. Since these substances would enter into the structure of only a few molecules in the cell, therefore only very small quantities would presumably be required. It is now apparent that the enzyme-coenzyme systems may be included among the cell constituents for which special structural material is needed.

Of the definite compounds now associated with growth-factor activity for microorganisms, *i*-inositol and β-alanine have not been shown to be components of an enzyme system, insofar as the writers are aware. The interpretation of their rôle in cellular metabolism must await further evidence. In the meantime, it is an interesting thought that the demonstration of the important part which these substances play in development of certain microorganisms may give a clue to their occurrence in some enzyme-coenzyme systems whose composition is now unknown.

The present knowledge of the growth factors, while fragmentary, permits a clearer idea of future lines of work which should

prove to be fruitful, and we are now better able to direct our efforts in solving the mysteries which still surround the growth requirements of many of the microorganisms. If one component of a coenzyme, such as nicotinic acid for example, is needed by a microorganism, perhaps two, three, four or more components of this or other systems may be required by still more exacting pathogens, or by some of the more fastidious types important to agriculture or to the fermentation industries. Following this line of reasoning, it might be assumed that some of the strictest parasites, which multiply only in the presence of living tissue or within living tissue cells, have lost a large measure of constructive ability in connection with their adaptation to such an abode. Such organisms might conceivably be unable to put together a needed organic catalyst even when supplied with its several component parts and perhaps will be found to need the intact, preformed constituents of a whole system.

SUMMARY

A number of attempts have been made to isolate the growth-promoting substances known to be widely distributed in animal and plant tissues. In many instances identification of the growth substances has not yet been accomplished, though some progress has been made in their separation. In other cases, however, several compounds of known chemical structure are now recognized as the active substances of tissue extracts. These are: hemin, *i*-inositol, thiamin, nicotinic acid and its amide, β-alanine, riboflavin and pyridine nucleotide phosphate (coenzyme or cozymase). With the exception of *i*-inositol, these compounds are needed only in very small amounts.

The microorganisms for which one or more of these compounds must be supplied are: *H. influenzae* and related types, propionic and lactic acid bacteria, staphylococci, diphtheria bacillus, dysentery bacilli, certain of the true fungi including some of the yeasts, and certain protozoa. On substitution of the required compounds for tissue extracts, it is now possible to cultivate a number of these types in synthetic media.

Another substance, biotin, has been obtained in crystalline

form as the methyl ester. Others have been obtained in a relatively pure state: the "sporogenes vitamin," pantothenic acid, the "L" fraction for lactic acid bacteria.

Microorganisms requiring the foregoing compounds are unable apparently to synthesize them. There is increasing evidence that other less fastidious types are able to construct them from simpler substances. The various constructive abilities of different organisms are significant with respect to symbiotic and other mutual relationships.

With the exceptions of *i*-inositol and β-alanine, the accessory factors are known to enter into the structure of enzyme-coenzyme systems catalyzing oxidation processes.

The growth-promoting effect of tissue extracts cannot be explained solely on the basis of the inorganic salt content or an alteration in the physical properties of the culture medium.

It is significant that recent work has tended to show the close relationship between the nutrition and metabolism of microorganisms and the higher forms of plant and animal life.

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